STUDIES ON HOLOTHURIAN COELOMOCYTES. II. THE ORIGIN OF COELOMOCYTES AND THE FORMATION OF BROWN BODIES

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Few accounts of the origin of holothurian coelomocytes are available. Cuénot (1897) and Hatanaka (1939) reported that coelomocytes originate in the hemal ring and vessels. Prosser and Judson (1952) mentioned the possibility that coelomocytes of *Stichopus* may originate in the hemal vessel. Hérouard (1889) indicated that coelomocytes originate in the tissues of the respiratory trees. Endean (1958) indicated that homogeneous amebocytes (lymphocytes) originated from the lining epithelium of the respiratory trees in *Holothuria leucospilota,* migrate into the coelomic fluid, and differentiate into morula cells and possibly other coelomocyte types. Hausmann (1931) claimed amebocytes were derived from the coelomic epithelium (peritoneum). Ohuye (1938) proposed that the category of coelomocytes known as lymphocytes (see Hetzel, 1963) represented primitive pluripotent cells from which all other coelomocyte types are derived. Théel (1920) indicated crystal coelomocytes were derived from amebocytes.

No reports describing the formation of holothurian brown bodies are known. The present study is an attempt, first, to clarify the origin of coelomocytes and their patterns of differentiation, and second, to provide some knowledge of the formation of brown bodies in holothurians.

Materials and Methods

In hope of increasing the rate of coelomocyte production, fifteen specimens of each of the following four species of holothurians were bled daily for five consecutive days: *Cucumaria miniata, Eupentacta quinquesemita, Parastichopus californicus,* and *Psolus chitonoides.* Perivisceral coelomic fluid was withdrawn with a hypodermic syringe and needle. As much as 3 cc. of coelomic fluid could be withdrawn from a large *Cucumaria* during the first bleeding, but increasingly smaller amounts of coelomic fluid were obtainable during subsequent bleedings. Bleeding did not cause evisceration in the species studied. Following the series of bleedings, the holothurians remained undisturbed for two days. On the eighth day, samples of coelomic fluid were withdrawn from experimental animals and from control animals that had not been previously bled. Smears of this coelomic fluid were vitally stained with toluidine blue and Janus green B, as described by Hetzel (1963). Other smears were fixed with osmic acid or formalin vapors for 30 to 60 minutes, air-dried, and stained in a variety of ways. The periodic acid-Schiff

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(PAS) technique, the Alcian blue method, and the ribonucleic acid extraction technique, as outlined in Pearse (1960) on page 832, 838, and 916, respectively, were used. Some smears were stained with the mercuric chloride bromphenol blue technique of Mazia, Brewster and Alfert (1953), or with hematoxylin and eosin.

Both experimental and control animals were sacrificed on the eighth day. Samples of the body wall and all major organs were fixed with Bouin's fixative, dehydrated in ethyl alcohol, embedded in paraffin, and sectioned at 10 microns. Sections were stained with hematoxylin and eosin. All sections and smears were searched for sites of coelomocyte production.

Brown body formation was studied by means of injections of a 2% (W/V) suspension of lamp black (carbon) in filtered sea water into the perivisceral coelom of holothurians. Thirty specimens of *Cucumaria miniata* were injected with 2 to 4 ml. of the lamp black suspension, depending on the size of the organism; five specimens of *Eupentacta quinquesemita* and five of *Psolus chitonoides* were each injected with 1 ml. of the suspension. Each of five specimens of *Leptosynapta clarki* was injected with 0.5 ml. The injected animals were placed in individual fingerbowls or aquaria filled with sea water. One-ml. samples of coelomic fluid were withdrawn for examination after 30 minutes, 60 minutes, 2, 3, and 8 hours, and, thereafter, once daily for 30 days.

One *Cucumaria* was sacrificed every other day for a period of one month and after that at irregular intervals for up to six months. One *Psolus* was sacrificed each week for four weeks. The animals were dissected and searched for sites of carbon deposition. The entire digestive systems and respiratory trees were removed, and bleached and hardened in 95% ethyl alcohol for 12 hours before they were dissected and their contents and walls examined for the presence of black carbon deposits. No specimens of *Eupentacta* or *Leptosynapta* were sacrificed during the course of the experiment.

Results

No coelomocyte free in the coelomic fluid of experimental or control animals was ever observed in any stage of cell division. Samples of coelomic fluid taken from experimental animals showed no obvious differences from samples taken from control animals. Among the coelomocytes present in the samples taken from control and experimental animals were cells which could be arranged in graded series between lymphocytes and hemocytes, and between lymphocytes and amebocytes.

I. Series of cells intergrading between lymphocytes and hemocytes.

1. Larger than average oval to spherical lymphocytes, with an increased number of Janus green B-positive granules.
2. Cells as described above with one or more of the Janus green B-positive granules containing a clear vacuole.
3. Still larger lymphocyte-like cells, similar to those of type 2 above, but with hyaline cytoplasm tinged straw-yellow.
4. Cells slightly smaller than hemocytes and with straw-yellow cytoplasm, but without the typical yellow refractile granules of hemocytes.

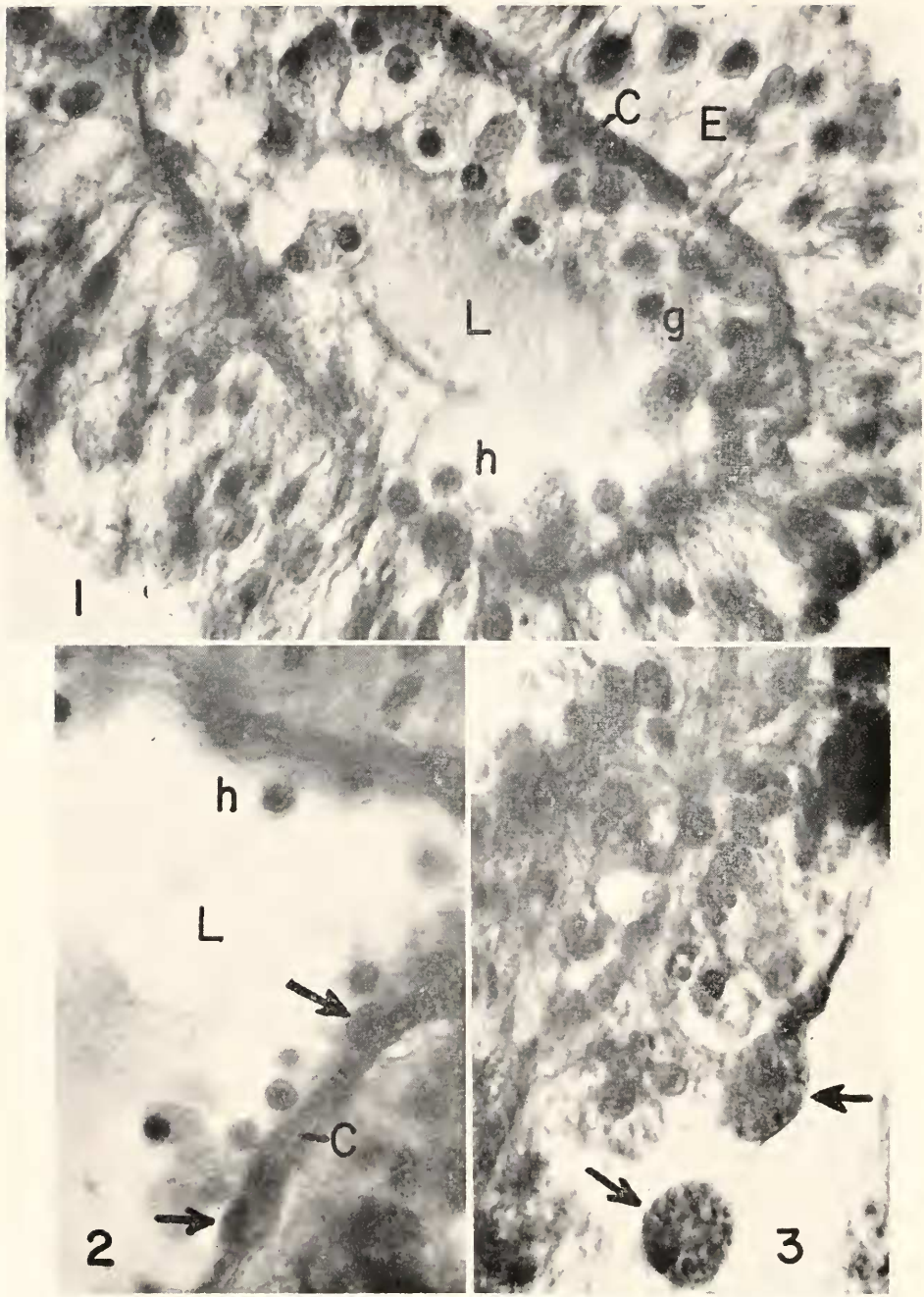

FIGURE 1. Cross-section of the dorsal hemal vessel of *Psolus chitonoides* (970×). C, connective tissue layer; E, external epithelium; L, lumen of vessel; g, lymphocyte with granular cytoplasm; h, lymphocyte with hyaline cytoplasm.

Cells belonging to Series I were observed in *Cucumaria* and *Eupentacta*, but not in *Parastichopus* or *Psolus*.

II. Series of cells intergrading between lymphocytes and amebocytes.

1. Unusually large lymphocytes with more than three filiform pseudopodia.
2. Larger cells with still more complicated pseudopodial systems and increased granulation.
3. Cells that approach amebocytes both in size and in degree of cytoplasmic granulation.

Cells belonging to Series II were found in all four species of holothurians examined.

No histological differences were noted between tissues taken from experimental animals and tissues taken from control animals. I found no evidence of the origin of coelomocytes from the peritoneum covering the inner surfaces of the longitudinal retractor muscles. No observations suggested that coelomocytes originate from the walls of the respiratory trees, nor from the body wall, the wall of the digestive tract proper, the polian vesicles, the water vascular system in general, nor from any part of the reproductive system.

Sections of the hemal vessels showed numerous lymphocytes within the lumina of the vessels (Fig. 1). The connective tissue layers of the hemal vessels and their associated mesenteries contained numerous mesenchymal cells with flattened, intensely staining nuclei. Mesenchymal cells located nearer the lumen of the hemal vessels contained oval, less intensely staining nuclei. Lymphocyte-like cells with oval nuclei and hyaline cytoplasm bulged from the connective tissue into the lumen of the hemal vessels (Fig. 2). Many of the latter cells formed a series exhibiting increasing size and granulation. These granulated cells can be arranged in a series intergrading with basophilic morula-like cells contained in the connective tissue layer of the mesenteries associated with the hemal vessels and the digestive tract (Fig. 3).

III. Series of cells intergrading between lymphocytes in the hemal vessels and basophilic morula-like cells.

1. Enlarged lymphocytes with homogeneous eosinophilic cytoplasm.
2. Enlarged cells as above, with finely granular eosinophilic cytoplasm; these cells were also observed in the connective tissue of the mesenteries associated with the hemal vessels.
3. Cells with amphiphilic staining properties and with granular cytoplasm located in the mesenteric connective tissue.
4. Amphiphilic cells in the connective tissue of the mesenteries with morphological features identical to those of morula cells.
5. Basophilic morula cells contained in the connective tissue layer of the mesenteries and free in the coelomic fluid.

FIGURE 2. Cross-section of hemal vessel of *Psolus chitonoides* (1292 ×). Arrows indicate oval nuclei of mesenchymal cells believed to be differentiating into lymphocytes. C, connective tissue layer; L, lumen of vessel; h, lymphocyte with hyaline cytoplasm.

FIGURE 3. Section of dorsal mesentery of *Psolus chitonoides* (970 ×). Arrows indicate basophilic morula cells.

Numerous eosinophilic morula cells were present throughout the connective tissues of the body other than the connective tissues of the gut, hemal vessels, and mesenteries adjacent to the hemal vessels and gut.

Spherules of morula cells fixed and stained at the moment of their release from cytolyzing cells were composed of an outer basophilic and metachromatic shell, and an inner core which was eosinophilic, but not metachromatic. The outer shell was PAS-positive and stained green with Alcian blue; the inner core stained blue when exposed to Mazia's bromphenol blue technique. Morula cells lost their basophilia following treatment with ribonuclease.

The injection of lamp black suspension into the coelomic cavity of holothurians apparently had little effect on the animals. Holothurians left undisturbed following the injection were observed to feed normally after one hour. Injected specimens were still normal and healthy six months after the injection.

Samples of the coelomic fluid withdrawn 30 minutes following the injection contained very few coelomocytes when compared to the number normally present. A sample taken one hour after the injection contained many more cells than did the previous sample, but the number was still clearly below normal. A few petaloid amebocytes contained engulfed carbon particles at this time, but these represented only a small per cent of the amebocytes present in the sample. In *Cucumaria,* carbon particles were occasionally observed within hemocytes. In many instances several amebocytes were observed clustered around masses of carbon particles.

The number of amebocytes containing engulfed carbon particles gradually increased up to the tenth to the fourteenth day following injection. By that time, almost every amebocyte contained carbon particles. Lymphocytes, morula cells, and crystal cells were never observed containing engulfed carbon.

Between the third and fourth weeks following injection the number of amebocytes containing carbon particles gradually decreased, and the number of amebocytes free of carbon particles increased. After six months an occasional amebocyte still contained particles of engulfed carbon. Amebocytes heavily laden with engulfed carbon particles did not produce large networks of filiform pseudopodia and, therefore, did not contribute actively to the formation of clots as did amebocytes free of carbon.

Most amebocytes with engulfed carbon particles also contained yellow granular material similar to the granular material composing brown bodies. Many spherical cells, completely filled with yellow granules and/or carbon particles, were observed.

Large masses of carbon were still found in the perivisceral coelomic cavity of *Cucumaria* after six months. As early as two days following the injection, noticeable accumulations of carbon were observed in the anterior portions of the water vascular system, especially in the polian vesicle, and in the ampullae of the anterior tube feet. Large masses of carbon were also located among the suspensor muscles of the cloaca, and along the junction of the dorsal mesentery and the gut. Carbon was accumulated in the same locations in *Psolus chitonoides.* After two weeks, carbon was also noted among the deposits of yellow granular material between the peritoneum and the wall of the sole of *Psolus.*

The walls of the excised gut and respiratory trees of *Cucumaria* and *Psolus* were searched for carbon deposits. Specks of carbon were occasionally found in the anterior and mid-gut regions two days following the injection, but no carbon was

detected in the lumina or walls of the respiratory trees. Occasionally, small amounts of carbon were found in the feces of *Cucumaria* and *Eupentacta*. After the second day, brown bodies composed partly of carbon were found in the aquaria containing injected *Cucumaria* and *Eupentacta*. Masses of amebocytes laden with carbon emerged from the bases of the tube feet of *Cucumaria*. Several specimens of *Cucumaria* were covered with carbon-laden mucus. After a period of three weeks the noticeable emission of carbon from the holothurians decreased and finally stopped.

Following the injection of lamp black, the normally clear coelomic fluid visible through the body wall of *Leptosynapta* appeared cloudy. Within two hours, however, the coelomic fluid was again clear and the carbon was concentrated in the ciliated funnels that line the junction of the mesenteries and the body wall of the animal.

The elimination of the carbon deposits from the ciliated urns of *Leptosynapta* occurred more rapidly than the elimination of carbon from bodies of the other holothurians studied. Within three days most of the carbon was gone from the ciliated funnels of *Leptosynapta*. Masses of carbon-laden amebocytes migrated from the ciliated urns through the body wall of *Leptosynapta* to the exterior.

Discussion

On the basis of observations made in the present study, one is led to support Ohuye's (1938) conclusion that the various coelomocyte types are possibly derived from a common source or stock of stem cells, and that all types of coelomocytes may be produced by direct transformation of the stem cells. Lymphocytes are probably the stem cells which differentiate into hemocytes, amebocytes, and morula cells. The source of crystal-bearing coelomocytes is unknown at the present time, but Théel (1920) believed that they differentiated from amebocytes.

The cells in Series I may be interpreted as lymphocytes undergoing differentiation into hemocytes. This view is further supported by the finding that these intermediate stages are found in *Cucumaria* and *Eupentacta* which possess hemocytes, but not in *Parastichopus* and *Psolus* which lack hemocytes (Hetzel, 1963). The course of differentiation is presumed to be the following: lymphocytes free in the coelomic fluid enlarge and produce increased numbers of Janus green-stainable granules; as hemoglobin synthesis occurs, the cytoplasm becomes straw-yellow in color and the large yellow refractile granules typical of holothurian hemocytes appear; the refractile granules appear to be derived from the Janus green-positive cytoplasmic granules.

Cells in Series II may be interpreted as lymphocytes differentiating into amebocytes. Lymphocytes are presumed to increase in size and produce more complex systems of pseudopodia as they differentiate into amebocytes.

No evidence was found in the present study to indicate that morula cells differentiated from lymphocytes free in the coelomic fluid as Endean (1958) proposed. Observations indicate that lymphocytes in the hemal vessels and closely associated gut mesenteries may form morula cells. The course of this differentiation is believed to be as follows: the hyaline cytoplasm of these lymphocytes gradually becomes granular; the granules gradually increase in size and are transformed

into the spherules typical of morula cells; during the transformation of the granules into spherules, the cytoplasm of the lymphocytes changes from eosinophilic to amphiphilic to the basophilic condition typical of morula cells located in the connective tissue framework of the mesenteries and morula cells free in the coelomic fluid.

The basophilia of morula cells was removed by ribonuclease digestion, and the cores of the spherules of the morula cells appear to be protein, as indicated by their positive staining reaction to Mazia's bromphenol blue technique. These observations indicate that morula cells may play an important role in amino acid uptake from the digestive tract and in protein synthesis and storage. If the morula cells indeed do function in amino acid uptake and in protein synthesis, as available evidence suggests, their possible site of origin in the hemal vessels, which are so closely related to the digestive tract, would be especially significant. Eosinophilic morula cells found throughout the connective tissue layers of holothurians possibly represent morula cells that have completed their synthetic activities and await an as yet undescribed function.

Endean (1958) reported that spherules of morula cells of *Holothuria leucospilota* also consisted of an inner proteinaceous core and an outer metachromatic shell believed to be, at least in part, mucopolysaccharide. The present study confirms his observations of the structure of the spherules of morula cells, and supports Endean's theory that they differentiate from lymphocytes. In species studied in the present report, however, morula cells appear to differentiate in close association with the hemal vessels, and not in the perivisceral coelom, as Endean described.

None of the observations made in the present study contribute to an understanding of the formation of crystal cells in holothurians. The crystals are not able to withstand the effects of fixation and preparation of tissue sections. Possible stages in the formation of crystal cells were likely destroyed in the prepared tissues, and only fully differentiated crystal cells were observed in living material. Théel (1920) claimed that crystal cells were derived from ameboid cells.

The fact that crystal cells, hemocytes, and morula cells are able to produce pseudopodia of some form (Hetzel, 1963) lends credibility to the hypothesis that these cells may all be derived from a common form that also gives rise to amebocytes.

The site of origin of lymphocytes proved to be a problem not easily resolved. Hausmann (1931) maintained they originate from cells of the peritoneal lining of the coelom. This may be so, but would be difficult to prove conclusively. Ohuye (1938) does not rule out the possibility that mesenchymal connective tissue cells may contribute to the formation of lymphocytes in holothurians.

Observations made in the present study indicate that at least some of the lymphocytes may differentiate from mesenchymal connective tissue cells in the walls of the hemal vessels. I believe that lymphocytes more likely are derived from mesenchyme than from epithelial cells such as those that make up the peritoneum. My observations suggest that lymphocyte-like cells in the hemal vessels possibly differentiate into morula cells. Other lymphocytes conceivably could migrate from their site of origin into the coelomic cavities, and there differentiate into hemocytes and amebocytes.

Schinke (1950) studied the origin and differentiation of coelomocytes of the echinoid, *Psammechinus miliaris*. Schinke reported that white amebocytes (lymphocytes?) were formed by direct transformation of cells in the connective tissue matrix of the calcareous tests. Schinke stated that the formation of the coelomocytes never involved mitosis, nor did the coelomocytes themselves ever undergo cell division. Holothurian coelomocytes have not been observed in cell division.

In light of Schinke's study, and the observations made in the present investigation, it seems reasonable to assume that lymphocytes are produced from mesenchymal connective tissue. The observations of Hatanaka (1939) of cells in the hemal vessels of holothurians also support this hypothesis.

Cuénot (1897) defined a lymphoid organ in invertebrate animals as an organ closely related to the blood cells and coelomocytes, and one that is composed of amoeboid cells or produces them. Cuénot described a lymphoid organ as a connective tissue framework in which are found many corpuscles identical to those that occur free in the body fluid. On the basis of Cuénot's definition, and on suggestions proposed in the present study, the hemal system of holothurians may be regarded as lymphoid tissue that functions in part to produce cells from which the coelomocytes are derived. Cuénot, himself, considered the hemal ring of holothurians as a lymphoid organ. The rete mirabile, an extension of the hemal system, may also function in part in the formation of coelomocytes.

On the basis of a survey of the coelomocytes of various invertebrate animals, including two species of holothurians, Ohuye (1938) derived lymphocytes from mesenchymal and/or peritoneal cells. Ohuye derived erythrocytes (hemocytes), amphiphilic, eosinophilic, and basophilic granulocytes (morula cells), and pigmented leucocytes from lymphocytes.

The existence of holothurian coelomocytes possessing characteristics intermediate between lymphocytes and hemocytes, morula cells, and amebocytes, lends support to Ohuye's views of coelomocyte lineages in holothurians.

Amebocytes appear to have at least two functions in holothurians, as phagocytes and as active agents in the clotting reaction. These functions appear to compete against one another; two weeks after the injection of carbon into *Cucumaria*, when the amebocytes were at peak activity in disposing of injected carbon, the efficiency of the clotting mechanism was low. Later, when more amebocytes were again free from the chore of carbon elimination, the efficiency of the clotting reaction returned.

The inability of carbon-laden amebocytes actively to contribute to the formation of a clot may easily be explained. As the amebocytes became filled with carbon particles, they became less ameboid and were eventually transformed into spherical cells laden with carbon; some also contained yellow granular material. Amebocytes partially filled with carbon granules were impaired in their ability to produce the extensive networks of filiform pseudopodia necessary for clot formation.

Observations of hemocytes containing small quantities of engulfed carbon were especially interesting. This is the first report of such an observation. The weakly phagocytic nature of hemocytes could indicate some relationship between hemocytes and amebocytes and lends further support to Ohuye's (1938) scheme of coelomocyte lineages.

The inclusion of carbon within brown bodies supports the hypothesis that brown

body material must be considered a product, if not truly excretory, at least of no use to the holothurian. The fact that carbon is engulfed by amebocytes that often contain yellow granules, and the fact that the carbon-laden cells are accumulated in the same region of the body where masses of yellow granules were found, would seem to indicate that the injected carbon particles and yellow granular material are both disposed of by phagocytic amebocytes. The additional fact that yellow granules and carbon appear together in brown bodies would also indicate that brown bodies are indeed a material that is actively accumulated by amebocytes and eliminated by the holothurian. The presence of brown bodies in holothurians which both possess and lack hemocytes would seem to indicate that these granules are not necessarily related to hemoglobin synthesis or degradation.

The lack of carbon in any of the morula cells or crystal cells would seem to indicate that these cells are very specialized and have lost all powers of phagocytosis.

One other speculation may be made regarding the phagocytic nature of amebocytes and their role in removing unwanted materials from the body of holothurians. Hetzel (1963) reported amebocytes and brown bodies containing one or more hemocytes, morula cells, or crystal cells. These observations suggest that the elimination of old or damaged coelomocytes from the bodies of holothurians might be performed in a manner similar to the elimination of carbon or yellow granular material. If this were true, the constant loss or elimination of these coelomocytes in brown body material would require the continued replacement of these coelomocytes and, therefore, the continued production of new coelomocytes throughout the life of the holothurian.

Summary

1. Coelomocyte and brown body production were studied in four species of holothurians, *Cucumaria miniata*, *Eupentacta quinquesemita*, *Parastichopus californicus*, and *Psolus chitonoides*.

2. Available evidence suggests that at least some, if not all, lymphocytes possibly originate from mesenchymal cells in the hemal vessels of holothurians, and possibly later differentiate directly into hemocytes, amebocytes, and morula cells.

3. The coelomic fluid of *Cucumaria* and *Eupentacta* contains coelomocytes which can be arranged in a series intergrading between lymphocytes and hemocytes. These cells possibly represent stages in the transformation of lymphocytes into hemocytes.

4. *Parastichopus* and *Psolus*, which lack hemocytes, do not possess the series of cells interpreted as stages in hemocyte differentiation.

5. All four species of holothurians studied possess coelomocytes which can be arranged in a series intergrading between lymphocytes and amebocytes. These cells possibly represent stages in the differentiation of lymphocytes into amebocytes.

6. Granular lymphocyte-like cells located in the hemal vessels of the four species studied possibly represent stages in the differentiation of lymphocytes into morula cells.

7. *Cucumaria miniata*, *Eupentacta quinquesemita*, *Psolus chitonoides*, and *Leptosynapta clarki* were injected with suspensions of lamp black in sea water.

8. The carbon particles were engulfed by amebocytes which often also contained granules similar to the granules found in brown bodies.

9. Carbon particles were accumulated in the same regions of the body as brown body material.

10. Brown bodies composed partly of carbon particles were eliminated by the holothurians.

11. Amebocytes are believed to play a role in the accumulation of brown body material and in the production of brown bodies.

12. Carbon particles were also concentrated in the ciliated funnels or urns of *Leptosynapta clarki*.

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